UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERISATION OF A LACTOCOCCAL PLASMID AND PRELIMINARY CONSTRUCTION OF A LACTOCOCCUS - E. COLI SHUTILE VECTOR

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By

ERNIE EILEEN RIZLAN ROSS

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

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Chairperson: **Raha Abdul Rahim, Ph.D.**

Faculty: **Food Science and Biotechnology**

A total of 38 isolates from the ceacal content of two weeks old chicks were positively identified as *Lactococcus lactis* using API 50 CH Identification kit (bioMérieux, France). Plasmid analysis was performed on all 38 isolates. Seven isolates (A105, B61, B106, C5, C62, C119 and D41) were found to carry small sized plasmid. Antibiogram of the seven isolates against seven antibiotics showed that all of them being susceptible to ampicillin (10 μg) and penicillin (10 μg). All isolates were found to be resistant to streptomycin (10 μg), gentamycin (10 μg) and kanamycin (30 μg). Only isolate B61 showed susceptibility towards erythromycin whereas the others showed resistance. An erythromycin resistant plasmid from isolate C5 was successfully electro-transformed into a plasmidless *L. lactis* MG1363. The plasmid, designated pAJ01, was characterized by restriction endonuclease digestions. From the digestion results, a *Lactococcus – E. coli* shuttle vector was constructed by the ligation of plasmid pAJ01 with pUC19 at their *Eco*RI site. The recombinant plasmid designated pAJ02 was shown to be able to replicate well in both *E. coli* and *Lactococcus*. Both the plasmids pAJ01 and pAJ02 were

found to be highly stable in *Lactococcus* with the estimated stability of 100% and 98% respectively. The partial sequence of the plasmid pAJ01 was obtained and analysed for open reading frames (ORF). Two ORFs were identified and by using Basic Local Alignment Search Tools (BLAST) programme provided by National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), the two ORFs were identified as replication gene and erythromycin resistance gene. Full sequences of the two genes were obtained.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains.

PEMENCILAN DAN PENCIRIAN PLASMID *LACTOCOCCUS* DAN PEMBINAAN AWAL VEKTOR PENGANGKUT *LACTOCOCCUS* – *E. COLI*

Oleh

ERNIE EILEEN RIZLAN ROSS

Ogos 2001

Pengerusi: **Raha Abdul Rahim, Ph.D.**

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Sejumlah 38 pencilan bakteria dari sekum ayam berumur dua minggu telah dikenalpasti sebagai *Lactococcus lactis* menggunakan kit pengenalan API 50 CH (bioMérieux, France). Analisis plasmid telah dijalankan ke atas ke semua 38 pencilan bakteria. Tujuh pencilan bakteria (A105, B61, B106, C5, C62, C119 and D41) didapati mempunyai plasmid bersaiz kecil. Kerentanan terhadap tujuh jenis agen antimikrob menunjukkan kesemua pencilan bakteria adalah sensitif kepada ampisilin (10 μg) dan penisilin (10 μg). Kesemua pencilan bakteria juga menunjukkan kerentanan terhadap streptomisin (10 μg), gentamisin (10 μg) dan kanamisin (30 μg). Hanya pencilan bakteria B61 sahaja yang sensitif kepada eritromisin (15 μg). Plasmid yang rentan terhadap eritromisin, dinamakan pAJ01 dari pencilan C5 telah berjaya dimasukkan ke dalam sel perumah *L. lactis* MG1363. Pencirian plasmid pAJ01 telah dijalankan melalui kaedah penguraian enzim pembatas. Sebuah vektor pengangkut *Lactococcus* - *E. coli* telah dibina dengan pencantuman plasmid pAJ01 dan pUC19 untuk menghasilkan plasmid rekombinan yang diberi nama pAJ02. Plasmid rekombinan pAJ02 didapati sangat stabil di dalam

Lactococcus dengan anggaran kestabilan masing-masing adalah 100% dan 98%. Jujukan separa plasmid pAJ01 telah diperolehi. Jujukan separa tersebut menunjukkan kehadiran dua rangkaan bacaan terbuka. Dengan menggunakan program `Basic Local Alignment Search Tools' (BLAST) yang disediakan oleh `National Center for Biotechnology Information' (http://www.ncbi.nlm.nih.gov), kedua-dua rangkaan bacaan terbuka tersebut didapati adalah gen replikasi dan gen kerentanan eritromisin masing-masing. Jujukan penuh kedua-dua gen juga telah diperolehi.

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I certify that an Examination Committee met on 15th August 2001 to conduct the final examination of Ernie Eileen Rizlan Ross on her Master of Science thesis entitled "Isolation and Characterisation of a Lactococcal Plasmid and Preliminary Construction of a *Lactococcus – E. coli* Shuttle Vector" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

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Date: 13 DEC 2001

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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Ernie Eileen bt Rizlan Ross

Date:

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LIST OF ABBREVIATIONS

bp	-	basepair
$CaCl_2$	-	calcium chloride
Cm^R	-	chloramphenicol resistance
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediamine tetra acetic acid
Em	-	erythromycin
Em^R	-	erythromycin resistance
g	-	gram
h	-	hour
kb	-	kilobase
Km^R	-	kanamycin resistance
λ	-	lambda
L	-	litre
$MgCl_2$	-	magnesium chloride
μL	-	micro-litre
μg	-	micro-gram
mA	-	milliamphere
mg	-	milligram
mL	-	millilitre
mM	-	millimolar
min	-	minute

M	-	molar
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
OD	-	optical density
ORF	-	open reading frame
s	-	second
SDS	-	sodium dodecyl sulfate

CHAPTER I

INTRODUCTION

Lactic acid bacteria, including the members of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* have long been used in food fermentation processes. This includes a broad range of products derived from a variety of raw materials such as vegetables, cereals, meat and milk. The fermentation not only serves as preservation of the food but they also add to the development of flavour and texture of the products. Additionally, the *Lactococcus* and *Lactobacillus* have been reported to possess probiotic effects. These features explain the major economic importance of the lactic acid bacteria. The genus *Lactococcus* however is mainly used in dairy fermentation such as cheese and buttermilk production.

The genus *Lactococcus* itself has been studied extensively and is the genetically best characterised species of the lactic acid bacteria. Nonetheless, molecular studies on the *Lactococcus* are relatively new when compared to *Escherichia coli*. This is because the cell membrane of Gram-positive bacteria with its thick layer of peptidoglycan provides an effective barrier for DNA extractions and gene manipulations. In the late 1970s and early 1980s, researchers began to develop ways for gene transfer in Gram-positive bacteria including *Lactococcus* (De Vos and Simons, 1994). The development of DNA extraction protocols and gene transfer systems have opened doors for genetic manipulations in the *Lactococcus*.

Molecular studies on the *Lactococcus* have been focused on improving their abilities in dairy fermentation especially in cheese and buttermilk production (De Vos and Simons, 1988). These include improvement of starter cultures in order to improve the taste, texture and odour of cheese (Haandrikman *et al.*, 1989; Kondo, 1989; Rijnen *et al.* 2000). Other than that, efforts have been made to produce starter cultures that are insensitive to bacteriophages (Forde *et al.*, 1999).

Recently, the usage of the *Lactococcus* in molecular field has diversified (De Vos and Simons, 1994; Robinson *et al.*, 1997; Drouault *et al.*, 2000) giving new perspectives of the bacteria. They are generally regarded as safe (GRAS) organisms and together with the new advances in molecular field, *Lactococcus* are now being used in various fields from food fermentation to the synthesis of fine chemicals, pharmaceuticals, and other products. The bacteria are not only non-pathogenic but they also do not elicit any immune response and have been ingested throughout history placing it into the group of bacteria that have the potential to be used for vaccine delivery. *Lactococcus* exhibits the ability to express several homogeneous and heterogeneous proteins from both prokartotic and eukaryotic genes. However, one drawback in the molecular studies of the *Lactococcus* is that presently there are no commercially available plasmid vectors that can be obtained in the market. Therefore, there is a need to develop new plasmid vectors.

This study looks at a selected number of naturally occurring plasmids of *Lactococcus* isolated from chicken intestine, specifically the chicken caeca. The major objective of

this study is to construct a *Lactococcus – E. coli* shuttle vector. In order to achieve this objective, the following steps have to be undertaken:

- to isolate and identify *Lactococcus* spp. from the chicken intestine,
- to study the naturally occurring plasmid(s) from these isolates,
- to characterise a plasmid pAJ01 by restriction endonuclease digestion for the preliminary construction of a *Lactococcus – E. coli* shuttle vector, and
- to identify and analyse the replication gene and erythromycin resistant gene on the plasmid pAJ01.

CHAPTER II

LITERATURE REVIEW

Microflora of Chicken Intestine

The study of the microflora of chicken intestine has long been done since the early 1900s, unfortunately we still have very little understanding about it. One of the major problems frequently faced in many of these studies was in the recovery of the whole bacterial population from the intestine (Smith, 1965; Salanitro *et al.*, 1974a). It is generally known that the intestine is a source of a wide range of microbial species with various growth requirements. This fact complicates the selection of recovery media used in the isolation of microbes from the intestine. The recovery media has to be able to support the growth of most if not all of the bacterial population present in the intestine (Kelley, 1983). Isolation conditions also play a critical role in microflora studies of the intestine (Salanitro *et al.*, 1974b). In general, most of the bacterial species present in the intestine are mostly facultatively anaerobes and strict anaerobes. This is due to the low level of oxygen present in the gut intestinal tract. Thus, to look at the microbial composition of the intestinal tract, the isolation condition should be suitable in order to recover both the facultatively anaerobes and strict anaerobes bacterial species.

There are several reasons why scientists study the microflora of the chicken intestine. First, to study the development of intestinal microflora of healthy chickens from the moment the chicks hatched from their eggs until the chicks reached their age of maturity

where the intestinal microflora has stabilised (Barnes *et al.*, 1972; Salanitro *et al.*, 1974b).

Once the information has been gathered, then changes in the microflora of the chicken can be monitored especially in comparative studies such as the use of different types of animal feed and the addition of antimicrobial agents in the feed as growth promoters (Sarra *et al.*, 1992).

Since the intestine comprises of a wide range of bacterial species, it can be used as a natural source of certain bacterial species such as *Lactobacillus* and *Lactococcus*. Most of the studies on intestinal microflora of chickens were done actively during 1960s and 1970s. Unfortunately, the genus *Lactococcus* was only established in 1985 (Schleifer *et al.*, 1985). Prior to that the genus *Lactococcus* was placed in the *Streptococcus* family under the Lancefield serological Group N making it difficult to trace any early studies on the lactococci.

Lev and Briggs (1956ab) were one of the earliest successful researchers to study the microflora development in chicken intestine. They looked at newly hatched chicks taken directly from the incubator and found that these chicks hardly had any microorganism in the crop, gizzard duodenum and ileum as what they have expected. However, they were able to detect dense microflora in the caeca of the chicks, which was mainly dominated by the *Clostridium* sp. Only an hour after hatching, Lev and Briggs (1956a) observed a rapid establishment of microorganisms in all parts of the intestinal tract. Interestingly, after 12 – 48 h post hatching, it was reported that *Escherichia coli* and *Streptococcus* sp. have started to dominate while the number of *Clostridia* spp. was observed to decrease

even though the total bacterial count increased through time. Nevertheless, with age, *E. coli* and *Streptococcus* spp. counts decreased in all parts of the intestine with exception of the caeca.

Salanitro *et al.* (1974a,b), using a non-selective medium developed for isolating rumen anaerobic bacteria, isolated at least 11 groups of bacteria from the caeca of chickens. They found that 90 % of the 298 isolates represented species of anaerobic Gram-negative cocci, facultatively anaerobic cocci and streptococci, *Peptostreptococcus*, *Propionibacterium*, *Eubacterium*, *Bacteroides* and *Clostridium*. A total of 17.5 % represents two types of facultatively anaerobic bacteria (Gram-positive cocci and *E. coli*).

In a subsequent study, Salanitro *et al.* (1978) found that the streptococci, lactobacilli and *E. coli* accounted for about 60 – 90 % of the bacteria in the duodenum, and upper and lower ileum. Predominant anaerobes recovered from the caeca included Gram-positive cocci, *Eubacterium*, *Clostridium*, *Gemmiger*, *Fusobacterium* and *Bacteroides* species.

Unfortunately, microflora studies on the chicken intestines have dramatically reduced to almost none in the 1980s. Be that as it may, from the studies presented, we can observe that the results on the microflora of the chicken intestine vary in some cases. This is due to many factors such as isolation techniques and recovery medium used. However, most studies confirmed that the intestinal tract of newly hatched chicks is fairly sterile except for the caeca and that as soon as the chicks started feeding, the microflora rapidly developed. Through the findings from the studies discussed, it was also agreed that the

caeca of the chicken intestinal tract has the highest bacterial count compared to any other regions of the intestine.

Lactococcus

General Information

The genus *Lactococcus* was established by Schleifer *et al.* (1985) for the lactic streptococci, S. *lactis* and *S. cremoris*. Bergey's Manual® of Determinative Bacteriology (1975) described this genus as spherical or ovoid in shape with the size of 0.5 – 1.2 X 0.5 – 1.5 μm. Lactococci usually appears in pairs or short chains in liquid media. The lactococci are non-motile, catalase negative, oxidase negative, facultatively anaerobic Gram-positive cocci that do not form endospores. This genus is chemoorganotroph, meaning that it relies on chemical compounds for energy and uses organic compounds as a source of electrons. The lactococci are able to ferment a number of carbohydrates but produces mainly L (+) – lactic acid without any gas production. The optimum growth temperature for this genus is at 30°C. Another characteristic of lactococci is that they can grow between 10°C and 40°C but the cells rapidly lose their viability if they are subjected to temperatures greater than 45°C. They are also of Lancefield serological Group N. Generally, the lactococci are usually found in dairy and plant products.

Taxonomy

Due to the similarities between *S. lactis* and *S. cremoris*, the 9th edition of Bergey's Manual® of Systematic Bacteriology (1986) grouped *S. lactis*, *S. lactis* ssp. *diacetylactis*, and *S. cremoris* into one species; *S. lactis*. Garvie and Farrow (1982) suggested the subspecies designation of *S. lactis* ssp. *lactis*, *S. lactis* ssp. *cremoris*, and *S. lactis* ssp. *diacetylactis*. However, based on nucleic acid hybridisation studies (Ludwig et. *al.*, 1985), immunological relationships of superoxide dismutase, lipoteichoic acid structures, lipid patterns, and fatty acids and menaquinone composition, Schleifer *et al.* (1985) proposed that the lactic streptococci be classified within a new genus, *Lactococcus*. The *International Union of Microbiology Society* approved the *Lactococcus* genus in 1986 (Anonymous, 1986). The new nomenclature now designates *S. lactis* and *S. lactis* ssp. *diacetylactis* as *Lactococcus lactis* ssp. *lactis* and *S. cremoris* as *Lactococcus lactis* ssp. *cremoris*. Sandine (1988) suggested that strains of *Lactococcus lactis* ssp. *lactis*, which utilise citrate to for diacetyl, to be termed *Lactococcus lactis* ssp. *lactis* var. diacetylous. The proposed terminology would be quite beneficial because citrate-fermenting lactococci are so widely used by the dairy industry.

Antimicrobial Resistance in *Lactococcus*

Antibiotic susceptibility tests have been extensively used as a method of characterisation of the bacterial species. This method was however mainly used on pathogens with the